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Cosmos caudatus extract/fractions reduce smooth muscle cells migration and invasion in vitro: A potential benefit of suppressing atherosclerosis

Said Moshawih [a,*], Manraj S. Cheema [a], Zaid O. Ibraheem [b], Nirmala Devi Tailan [a], Muhammad Nazrul Hakim [a]

[a] Department of Biomedical Sciences, Faculty of Medicine and Health Science, Universiti Putra Malaysia, Selangor, Malaysia
[b] Department of Human Anatomy, Faculty of Medicine and Health Science, Universiti Putra Malaysia, Selangor, Malaysia

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ABSTRACT

Background: Cosmos caudatus Kunth is a medicinal herb used traditionally in Latin America and South East Asia to retard aging, rigidify bones and for several cardiovascular uses.

Objective: Is to assess C. caudatus extract/fractions' antioxidant and vascular smooth muscle cells (VSMC) migration and invasion inhibition capacity in vitro.

Methods: Cosmos caudatus shoots were extracted by cold maceration in 50% ethanol to produce crude (CEE), and then the extract was fractionated to butanol (Bu.F), and aqueous fractions (Aq.f). Phenolics and saponins were quantified in extract and fractions by colorimetric methods and their antioxidant capacity was assayed in four different tests. Cytotoxic effect and safety level concentrations were determined for the fractions by using MTT assay. Migration and invasion inhibitory potential were measured in vitro at three different concentrations equivalent to (IC_{10}, IC_{25}, and IC_{50}). Finally, invasion inhibitory index was calculated to obtain the best fraction(s) that show(s) the highest ratio of cell invasion inhibition to the total cell migration inhibition.

Results: Butanol fraction yield was the lowest; nevertheless, its phytochemical contents, antioxidant activities as well as its potency were the highest. Unlike other fractions, Bu.F was strongly correlated with all antioxidant assays experimented. In addition, it has the highest inhibitory effect at IC_{25} against VSMCs migration and invasion that accounts for 53.93% and 59.94% respectively. Unexpectedly, Bu.F and CEE at IC_{10} displayed the highest invasion inhibitory index (approx. 68%).

Conclusion: Butanol fraction of C. caudatus offers a potentiality for the discovery of new leads for preventing atherosclerosis.

Introduction

Atherosclerosis is a group of cardiovascular diseases (CVD) characterized by a chronic inflammatory condition that affects arterial blood walls, leading to lipid deposition under tunica intima and finally, the atherosclerotic plaque formation.[1] According to International Atherosclerosis Society (IAS), it comprises coronary and peripheral artery diseases, in addition to strokes and myocardial infarctions. Atherosclerotic cardiovascular diseases account for 40–50% of deaths in the developed countries, which are more than all cancer forms' mortalities. Similarly, atherosclerosis has a growing number of morbidities and mortalities in developing countries as reported by a survey done on behalf of the European Atherosclerosis Society.[2]

The physiological migration and invasion takes place by the de-differentiated VSMCs to perform developmental processes and in response to vascular injury. When those de-differentiated VSMCs fail to switch to the differentiated phenotype after angiogenesis or tissue repairing, a pathological cell migration/proliferation (synthetic cells) will lead to atherosclerosis development. Differentiated VSMCs in normal blood vessels function as contractile cells that regulate vascular blood flow and diameter.[3] It is noteworthy that the key step in atherosclerosis is the migration of de-differentiated SMC as well as the secretion of protease enzymes that break down the ECM in order to reach the intima layer in a process called invasion.[4] Therefore, it can be considered that measuring cell migration and invasion inhibition capacity by drugs and plant extracts is a beneficial *in vitro* model to quantify their effects in reducing atherosclerosis.

In folk medicine, *C. caudatus* has been used in Southeast Asia for many uses, such as burns and muscular strains and spasms.[5] Moreover, people in the Philippines incubate *C. caudatus* leaves with rice in order to prepare yeast.[6] In addition to being consumed as an appetizer in salads and food, Malay and Javanese use *C. caudatus* leaves to tone up blood circulation, rigidify bones and for bad breath.[7] These traditional uses are ascribed to a wide range of phytochemical constituents screened in several studies such as phenolic acids, flavonoids, carotenoids, sesquiterpene lactone, vitamins and phenylpropanoids.[8–13] Although *C. caudatus* has demonstrated pharmacological benefits related to atherosclerosis risk factors, such as antihyperlipidemic, antihypertensive and antidiabetic effects,[14,15] its SMCs migration and invasion reduction effects have not yet been studied. The present study was primarily designed to determine cell migration and invasion inhibitory effects *in vitro* for *C. caudatus* extract and derived fractions, in addition to assessing their antioxidant activities and phytochemical contents.

Materials and methods

Plant extraction and fractionation

The fresh whole plant of *Cosmos caudatus* (Kunth) was procured from Selangor wet market, Malaysia in November 2014. Plant samples were identified by a botanist at the institute of bioscience (IBS), Universiti Putra Malaysia, Specimen voucher number is (SK 2574/14). Extraction and fractionation were carried out according to Chan et al.[16] with slight modifications. In brief, fresh shoots were picked up, oven-dried (Lab-Dryer Protech, FSD-380, Malaysia) for 3 h at 50 °C and mechanically powdered. The shoots powder was extracted twice for 48 h each with intermittent shaking by cold maceration in 50% ethanol, wherein the ratio of the plant to solvent was 1:15 (W/V), then filtered through Whatman filter paper no.1. Thereafter, the filtrate was evaporated under reduced pressure (Hei-VAP value Heidolph – Germany), and the resulting semisolid mass was freeze-dried (Virtis Benchtop K, USA) in order to produce the crude alcoholic extract (CEE). The Crude extract was defatted with n-hexanol for three times and fractionated with n-butanol and water, followed by evaporation under reduced pressure (Hei-VAP value Heidolph – Germany) for 5 times to produce the butanol fraction. At the end, the aqueous layer was freeze-dried to yield the aqueous fraction. The resulting extract/fractions were stored at −80 °C for further analysis.

Determination of total phenolic contents

Total phenolic content (TPC) was determined for each fraction by Folin-Ciocalteu (FC) reagent assay, as described by Chan et al.[17] Gallic acid was used as a standard. Briefly, 100 μL of (2 mg/mL)

samples were mixed with 500 μL of 10 times diluted (FC) reagent. After 1 min, 400 μL of (7.5 mg/mL) sodium bicarbonate solution was added and allowed to stand for 30 min at 40 °C (Binder incubator, BD53 – GmbH). Each sample was measured at 760 nm (Shimadzu, UV-1700, Japan). The TPC was calculated from the calibration curve Figure S1, and the results were expressed as (mg GAE/g extract).

Determination of total saponin contents

Total saponin content (TSC) was determined for each fraction according to Hiai et al.[18] Diosgenin was used as a standard. In brief, 100 μL of (10 mg/mL) sample was mixed with equal amounts of vanillin (8 mg/100 mL), and 1 mL of 72% (V/V) H_2SO_4 was mixed with them, and then incubated for 10 min at 60 °C. Optical density (OD) was recorded at 540 nm (Shimadzu, UV-1700, Japan). The TSC was calculated from the calibration curve Figure S2, and the results were expressed as (mg DE/g extract).

Determination of total steroidal saponin contents

Total steroidal saponin contents (TSSC) was determined for each fraction according to Baccou et al.[19] Diosgenin was used as a standard. Briefly, 500 μL of different concentrations for each fraction were mixed with 250 μL of anisaldehyde (0.5 mL/100 mL ethyl acetate). The resulting mixture was added to 250 μL sulfuric acid (50 mL/100 ethyl acetate). Subsequently, it was allowed to stand at room temperature for 30 min, and the absorbance was measured spectrophotometrically at 430 nm (Shimadzu, UV-1700, Japan). The TSSC was calculated from the calibration curve Figure S3, and the results were expressed as (mg DE/g extract).

Antioxidant activity assays

DPPH• scavenging activity assay

The DPPH• scavenging activity was measured for each fraction according to Chan et al.[20] with mild modifications. Briefly, 50 μL of (0.5 mg/mL) triplicate samples were added to 195 μL methanolic solution of (0.2 mM) 2,2-diphenylpicrylhydrazyl (DPPH) (Sigma–Aldrich, USA) in 96-well plate (TPP, Switzerland), and gently shaken for 1 min prior to be allowed to stand in the dark for 1 h at room temperature. Finally, absorbance was measured at 540 nm by a microplate reader (BioTek, Synergy H1, US). Trolox was used as a standard and the radical scavenging activity was expressed as mg TEAC/g extract (Figure S4).

ABTS•+ scavenging activity assay

ABTS•+ scavenging activity was measured for each fraction as explained by Chan et al.[17] with modifications. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS (Sigma–Aldrich, USA) test reagent was prepared by reacting 10 mL of both 7.5 mM ABTS solution with 2.45 mM potassium persulfate, and the resulting dark blue solution was allowed to stand in the dark at room temperature for 16 h. Subsequently, the solution was diluted with absolute ethanol to an absorbance of 0.7 ± 0.02 at 734 nm. Thereafter, 950 μL of ABTS test reagent was reacted with 50 μL of different concentrations of each fraction with mild shaking and then allowed to stand at room temperature for 1 h. Triplicate samples were transferred into a 96-well plate and the absorbance was measured at 734 nm by a microplate reader. Trolox was used as a standard and the radical scavenging activity was expressed as mg TEAC/g extract (Figure S5).

Ferric ion chelating activity assay

Ferric ion chelating activity was measured for *C. caudatus* extract/fractions as explained by Chan et al.[16] In brief, 1 mL of (0.5 mg/mL) samples were reacted initially with 50 μL of 2 mM

FeCl$_2$ (Sigma–Aldrich, USA), and followed by adding 100 μL of 5 mM ferrozine (Sigma–Aldrich, USA). The final mixture was vortexed and allowed to stand for 10 min at room temperature. Triplicate samples were transferred to a 96-well plate and the absorbance was measured at 562 nm. EDTA was used as a standard and the radical scavenging activity was expressed as (mg EDTA/g extract) (Figure S6).

Beta carotene bleaching activity assay

β-Carotene bleaching activity (BCB) was measured for *C. caudatus* extract/fractions as explained by Wettasinghe et al.[21] with minor modifications. Briefly, 3 mL of β-carotene solution (1.2 mg/mL chloroform) (Sigma–Aldrich, USA) was mixed with 180 mg linoleic acid and 1800 mg tween 20 (Fisher Scientific, USA), then they were shaken vigorously. Afterward, chloroform was evaporated rapidly using rotary evaporator (Hei-VAP value Heidolph – Germany) at 40 °C and 100 mBar. In order to prepare β-carotene–linoleic acid emulsion, 100 mL of distilled water was immediately added to the latter solution. Samples were tested by reacting 20 μL of each sample (2.5 mg/mL), with 1.5 mL of the emulsion and incubated for 60 min at 50 °C. Triplicate samples were transferred to a 96-well plate and the absorbance was measured at 470 nm. Trolox was used as a standard and the BCB activity was expressed as (mg TEAC/g extract) (Figure S7).

Cell culture

A-10 cell line is an adherent, non-differentiated vascular smooth muscle cells that have been derived from medial layer of embryonic rat aorta, but have significant resemblance with neointimal cells. At the same time, it has a myoblast morphological characteristic. (A-10) cell line was purchased from American type culture collection (ATCC® CRL-1476, Rockville, USA). It was maintained in Dulbecco modified Eagle medium supplemented with 10% FBS and 2% penicillin-streptomycin at 37 °C in 5% CO$_2$, cells were digested by 0.25% (W/V) trypsin/EDTA (Biowest, Nuaillé – France).

Cell viability assay (MTT)

VSMCs viability were quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. When culture flask reached 80% confluency, cells were harvested, and seeded at a density of 5×10^3/well in a 96-well plate and incubated overnight. Serially diluted *C. caudatus* extract/fractions in 200 μL media were added to each well and incubated for 24 h at 37 °C in 5% CO$_2$. Thereafter, 20 μL of MTT (Amresco, Ohio, USA) solution of (5 mg/mL PBS) was added to each well and incubated for 4 h at 37 °C in 5% CO$_2$. Subsequently, well contents were aspirated and 100 mL of DMSO (Amresco, Ohio, USA) was added to wells to dissolve formazan formed by cells, followed by gentle shaking for 10 min. Finally, the absorbance was measured by microplate reader (TECAN, Infinite 200 pro-Männedorf, Switzerland) at wavelength 570 nm and reference subtraction at 650 nm. The assay was repeated three times and each time four wells were allocated for each concentration.

Data revealed by this assay was used to calculate inhibitory concentrations (IC) for 10%, 25%, and 50% of the cell population for each extract/fraction from the dose–response curves.

Chemotaxis assay

Chemotaxis assay was carried out according to the manufacturer instructions (Merck Millipore Darmstadt, Germany), catalog number "ECM 508". When culture flask of VSMCs reached 80–85% confluency, it was starved with serum-free media for 18 h, and then harvested using trypsin/EDTA. Cells were treated with each extract/fraction in free–serum media for 30 min prior to being
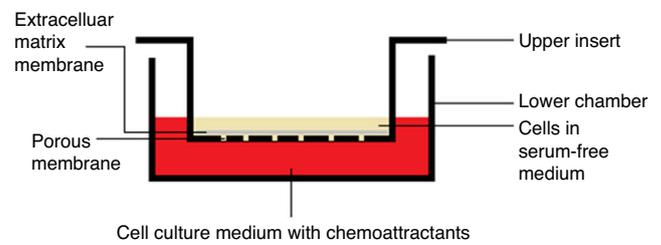
Fig. 1. Boyden Chamber Kit, where cells migrate from the upper insert to the lower chamber and invade the ECM membrane following a concentration gradient. In the case of migration kit, there is no ECM layer.

decanted in the insert. Each insert contains 300 μL free-serum media with $15–30 \times 10^4$ cells/insert.

After adding 500 μL media with 20% FBS to the lower chamber (24-well plate), insert was hanged on the lower chamber, covered and were incubated for 6 h at 37 °C and 5% CO$_2$ (Fig. 1). Subsequently, media with cells were pipetted out, followed by staining with dye provided with the kit for 20 min. Afterward, the interior surface of the insert was washed with water and dried with cotton-tipped swab prior to extracting the dye by the extraction buffer. Finally, 100 μL of the extracted stain was transferred to a 96-well plate and absorbance measured at 560 nm (TECAN, Infinite 200 pro-Männedorf, Switzerland).

The positive control insert was considered as 100% reference, where cells were left without treatment, while the negative control insert was considered as 0% reference, where the lower chamber was left without chemoattractants. The formula used to calculate migration inhibition percentage is:

Migration Inhibition Percentage

$$= 100 - \left(\left(\frac{(OD\, sample - OD\, negative\, control)}{(OD\, positive\, control - OD\, negative\, control)} \right) \times 100\% \right)$$

Chemoinvasion assay

Chemoinvasion assay was carried out according to the manufacturer instructions (Merck Millipore Darmstadt, Germany), catalog number "ECM 550". When culture flask of VSMCs reached 80–85% confluency, it was starved with serum-free media for 18 h, and then harvested by using trypsin/EDTA. Cells were treated with each extract/fraction in free-serum media for 30 min prior to being decanted in the insert. Each insert contains 300 μL free-serum media with $15–30 \times 10^4$ cells/insert.

After adding 500 μL media with 20% FBS to the lower chamber (24-well plate), insert was hanged on the lower chamber, covered and were incubated for 24 h at 37 °C and 5% CO$_2$ (Fig. 1). Using a cotton-tipped swab, non-invading cells, as well as the ECM gel, were gently removed from the interior surface of the inserts. This has been followed by staining with the dye provided with the kit for 20 min. Afterward, the interior surface of the insert was washed with water prior to extracting the dye by 10% acetic acid. Finally, 100 μL of the extracted stain was transferred to a 96-well plate and absorbance was measured at 560 nm. Positive and negative controls and calculations for the invasion inhibition percentage were exactly calculated as mentioned in the chemotaxis assay.

Statistical analysis

Data were expressed as a mean ± standard deviation for triplicate samplings. One way (ANOVA) was used to compare differences between groups. Post hoc comparisons were performed with Tukey test to indicate the significant variations between groups ($p < 0.05$).
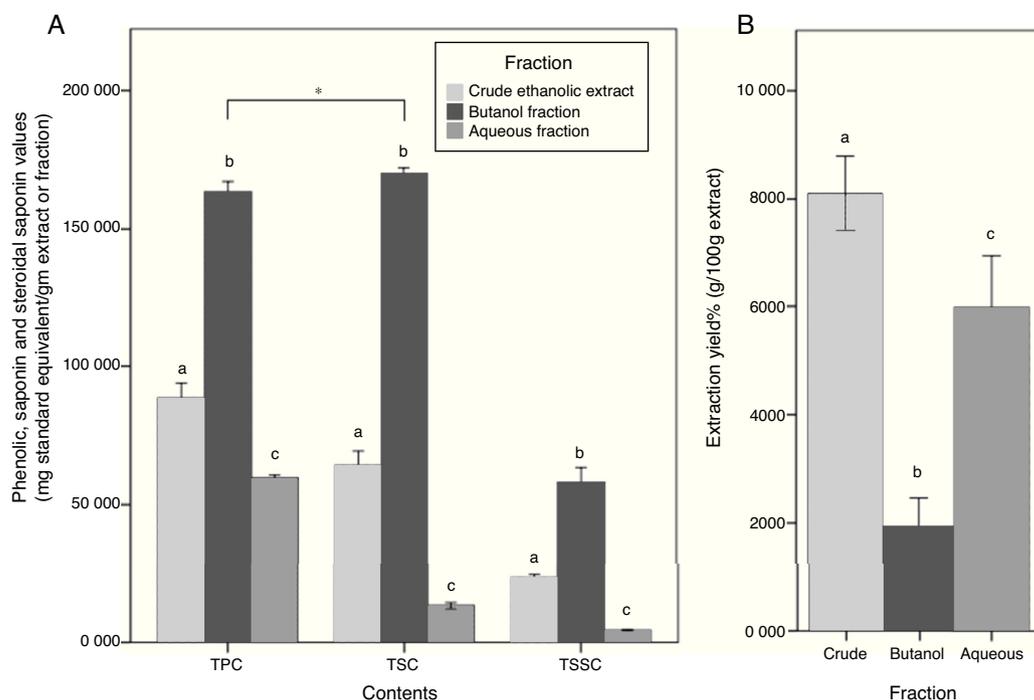
Fig. 2. (A) Total phenolic contents (TPC), total saponin contents (TSC) and total steroidal saponin contents (TSSC) for *C. caudatus* fractions. (B) The extraction yield for **crude (CEE), butanol (Bu.f) and aqueous (Aq.f) fractions**. Data were presented as a mean ± standard deviation, *n* = 3. a-c Different letters within the same group indicate significant difference (*p* < 0.05) according to ANOVA and post hoc with Tukey tests. *Significant difference between groups at (*p* < 0.05).

Pearson test was carried out to identify the correlation between phytochemical contents of the tested fractions and their antioxidant activities. In bar charts, different letters indicate statistically significant differences between groups.

Results and discussion

Yields of extraction and fractionation, total phenolics, saponin and steroidal saponin contents

Crude extraction yield was 8.10%. This yield was lower than what was reported in other studies carried out on *C. caudatus*, as they accounted for approximately 23%.[10,22] This can be ascribed to the implementation of heat reflux and sonication techniques. Both extraction methods are known to give higher yields compared to maceration. In particular, ultrasonic waves destroy the cell wall and allow solvents to penetrate inside and extract phytochemical constituents.[23]

Although butanol yield was the lowest among other fractions (1.9%), its TPC was approximately two times more than CEE, and almost 3 times more than the Aq.f (*p* < 0.05). Likewise, TSC of the butanol fraction was approximately 3 times more than the CEE, and 13 times more than the Aq.f (*p* < 0.05). In the TSSC determination, it has demonstrated the same trend of the previous results, all *C. caudatus* extract/fraction followed a descending order as follows Bu.F> CEE > Aq.f (*p* < 0.05) Fig. 2. For this reason, the Bu.F was considered the phenolics-saponins rich fraction (PSRF). Up to our knowledge, this is the first time saponins and steroidal saponins were quantitatively determined in *C. caudatus* extract/fractions. Remarkably, TSC was the highest content in Bu.F compared with TPC and TSSC (*p* < 0.05), and this might affect the antioxidant power of this fraction.

Total saponin content of the Bu.f was (0.83%). This value was comparable to saponin contents in some kinds of legumes which is known for high saponin contents such as soybean, chickpeas, and lentils (0.1–2%).[24] This indicates that *C. caudatus* leaves have

a considerable amount of saponins and can be used as a nutritive and economic resource that can be employed in the medicinal and cosmetic industry. In accordance with Kerem et al.[25] findings, the result of this study shows that water-butanol purification for saponins from *C. caudatus* ethanolic extract is the most suitable system to concentrate saponin contents. Specifically, the high extractability of this solvent system can be explained by that the diverse polarity indices of its components; water (9.0) and butanol (4.0), is compatible with saponins' amphiphilic nature that have both a hydrophobic nucleus and a hydrophilic glycoside. On the other hand, the high TPC that was yielded by butanol fractionation suggests that phenolics' polarity in *C. caudatus* constituents is compatible with the intermediate polarity of butanol and this is supported by low water extractability to phenolics. Besides, the high extraction and fractionation yields for the CEE and the Aq.f propose that high percentage of *C. caudatus* constituents are polar with a non-phenolic nature. Therefore, they have been substantially extracted by polar solvents; ethanol and water. Taken together, the high TPC, TSC, and TSSC in Bu.f distinctively propose the comprehensive extractability of n-butanol for phenolics and saponins in *C. caudatus*.

Saturated n-butanol was employed to concentrate saponins and phenolics from the crude extract of *C. caudatus*, and indeed, it had proved its effectiveness in this work in addition to other studies.[16,17] After fractionation with butanol, the fraction was subjected to evaporation under reduced pressure in order to rid all solvent residues, and consequently prevent any interference with cell homeostasis or antioxidant power in the further assays.

Antioxidant activities and their correlation with phytochemical contents

Antioxidant activities of the three fractions of *C. caudatus* were assessed by four different assays based on different mechanisms of action. Results obtained are shown in Fig. 3. DPPH and ABTS antioxidant activity assays were employed to evaluate the free radical
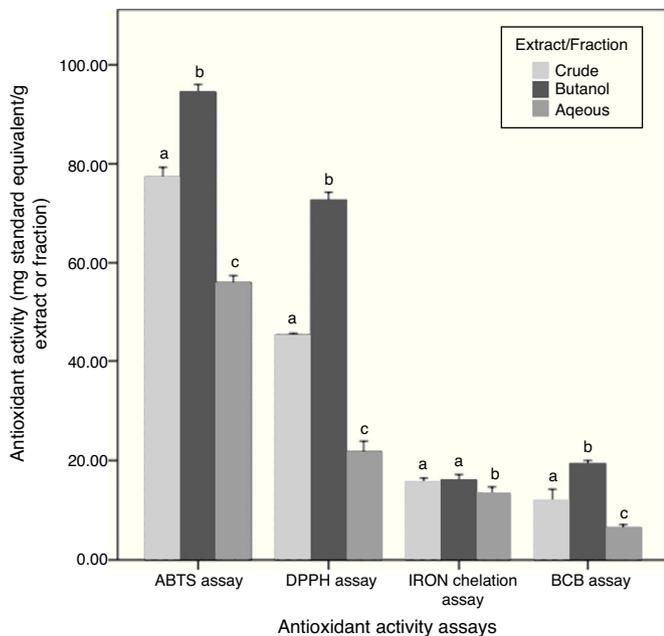
Fig. 3. Antioxidant activities for crude, butanol, and aqueous fractions derived from *Cosmos caudatus*. Data were presented as a mean ± standard deviation, $n = 3$. a-c Different letters within the same row indicate significant difference ($p < 0.05$) according to ANOVA and post hoc with Tukey tests.

Fig. 4. Cytotoxic effect of *C. caudatus* extract and fractions on the proliferation of A-10 cell line. Each data point represents the mean of three independent experiments ± SD with four replicates at each dosage. Control group represented 100%.

scavenging activities of *C. caudatus* extract/fractions. Both assays showed that Bu.f has the strongest antiradical activity, followed by CEE and the least power was found for the Aq.f ($p < 0.05$).

In addition to DPPH and ABTS assays, β-carotene bleaching activity is also considered an assay that measures the primary antioxidant activities. However, BCB was assayed to assess *C. caudatus* fractions' capacity in preventing lipid peroxidation.[26] Extract and fractions derived from *C. caudatus* were tested to minimize β-carotene oxidation by hydroxy peroxides generated from linoleic acid under heat conditions. In agreement with the above-mentioned results, Bu.f showed higher BCB activity (19.43 mg TEAC/g extract) than CEE (11.90), and the least activity was for the Aq.f (6.48) ($p < 0.05$).

Iron ion chelating activity was carried out in this work to determine the secondary antioxidant activity for *C. caudatus* extract/fractions. It is important to mention that secondary antioxidants work indirectly by retarding the oxidation process by many ways such as quenching singlet and triplicate oxygen, repairing primary antioxidants, chelating metal ions or by absorbing ultraviolet radiation.[27] In line with other antioxidant assays, Bu.f has demonstrated higher iron chelating strength (16.03 mg EDTA/g extract) than CEE (15.59) and Aq.F (13.39) ($p > 0.05$). This indicates that Bu.f is the strongest primary and secondary (preventive) antioxidant fraction. This can be attributed to that; n-butanol attracted both phenolics and saponins with multiple polarities ranging from non- to semi-polar compounds. Furthermore, Bu.f contains more saponin contents than phenolics ($p < 0.5$) Fig. 2, and this could substantiate the secondary antioxidant mechanism as well as the primary one.

A very strong correlation was observed between the phenolic content and DPPH, ABTS and BCB antioxidant activities (0.949–0.976), except for iron chelation activity as there was no significant correlation. On the other side, TSC and TSSC have strong correlations with all antioxidant activities including the iron chelation assay (0.692–0.989) (Table S1). Thus, it can be said that saponins and steroidal saponins have more contribution in the secondary antioxidant mechanism than phenolics. In addition to that, the high saponin contents can reasonably interpret Bu.f higher iron
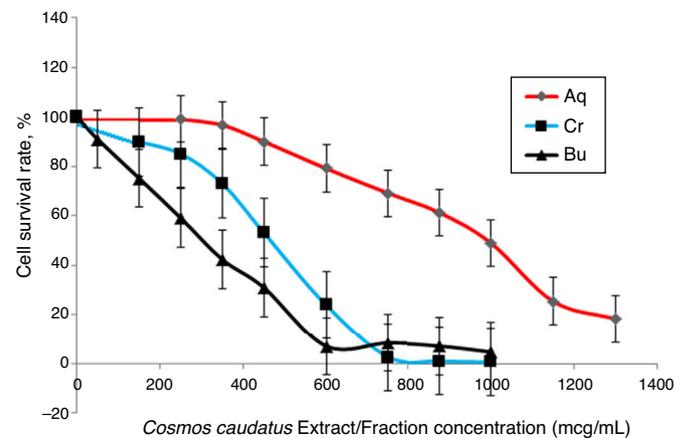
chelating activity than other extract/fractions. Moreover, butanol fraction as a whole has a strong correlation with all antioxidant assays tested in this work, which could not be applied to crude and aqueous extract/fraction.

Cell viability assay

The effects of Bu.f, CEE, and Aq.f on vascular smooth muscle cells viability were detected by MTT assay. A-10 cells were treated with different concentrations of *C. caudatus* extract and derived fractions for 24 h. A significant decrease in the cell viability after adding treatments have been shown in a dose- and time-dependent manner ($p < 0.05$) (Fig. 4). Table 1 shows the inhibitory concentrations for each extract/fraction at IC_{50}, IC_{25}, and IC_{10}. Butanol fraction described in the above-mentioned assays as a highly dense fraction of phytochemicals has the highest cytotoxic effect that decreases the viability of A-10 cells, thus, it has the lowest IC values, followed by CEE and lastly the Aq.F.

Potent fraction generally reduces cell viability in the MTT assay in a lower concentration than non-potent ones. Similarly, it gives more pronounced pharmacological effects at lower doses that are usually used to treat cells in culture.[28,29] However, butanol and crude fraction/extract in this study cannot be considered cytotoxic due to that the American national cancer institute states that plant fractions are considered cytotoxic to cancer cells when their IC_{50} is less than 20 μg/ml.[30] Moreover, concentrations are commonly employed in pharmacological assays should demonstrate a slight reduction in cell viability that does not reach a statistical significance.[31] Exceptionally, we tested three concentrations of *C. caudatus* extract/fractions (IC_{10}, IC_{25}, and IC_{50}), which could cause an effect on cell viability, especially at IC_{25} and IC_{50} in order to shed some light on the effects of highly concentrated phytochemicals on cell behavior *in vitro*. Further explanation for the effect of each concentration on cells in the following pharmacological assays will be discussed later in this article.

Table 1
Cosmos caudatus extract and derived fractions inhibitory concentrations for 10, 25, and 50% of the cell count.

Fraction	IC_{50} (μg/mL)	IC_{25} (μg/mL)	IC_{10} (μg/mL)
Butanol	313.96 ± 13.52 [a]	153.16 ± 10.07 [a]	56.67 ± 9.47 [a]
Crude	460.78 ± 25.63 [b]	266.63 ± 49.83 [b]	147.77 ± 22.46 [b]
Aqueous	949.20 ± 14.24 [c]	629.09 ± 25.13 [c]	437.02 ± 43.95 [c]

a-c Different letters within the same column indicate significant difference ($p < 0.05$).
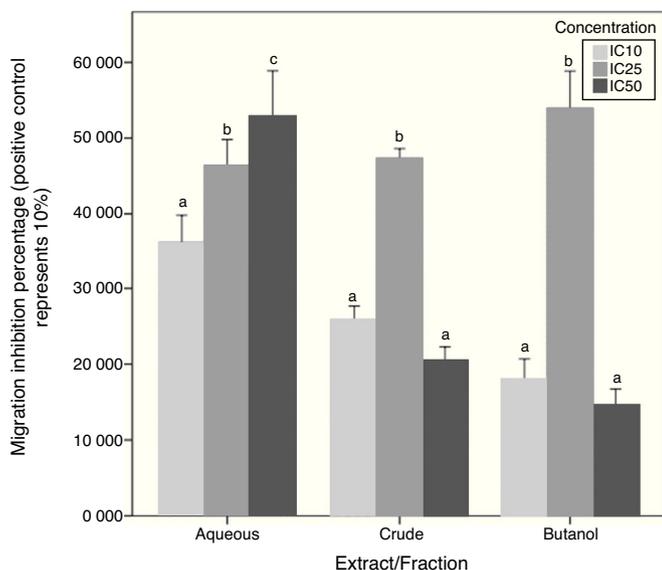
Fig. 5. Migration inhibition capacity for *C. caudatus* CEE, Bu.F, and Aq.F. Different letters within the same fraction indicate significant differences ($p < 0.05$).
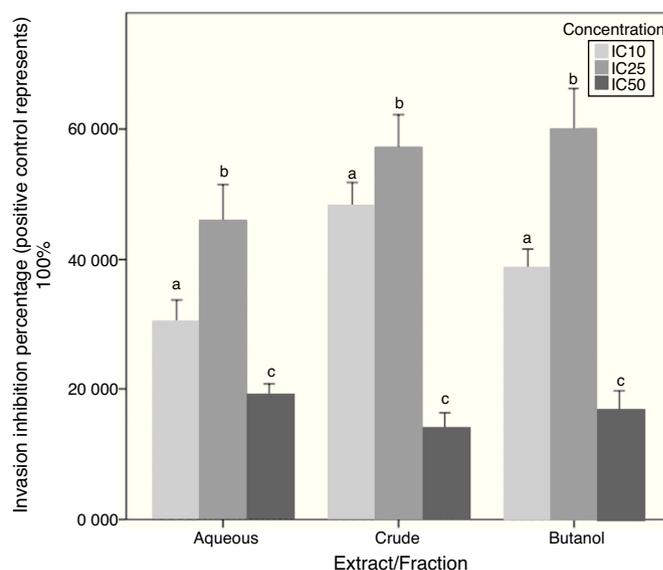

Fig. 6. Invasion inhibition capacity for *C. caudatus* CEE, Bu.F, and Aq.F. Different letters within the same fraction indicate significant differences ($p < 0.05$).

Chemotaxis inhibition activity

A-10 cells migration inhibition activity for CEE, Bu.f, and Aq.F of *C. caudatus* at three different concentrations (IC_{10}, IC_{25}, and IC_{50}), were determined by this assay. The increase of migration inhibition of VSMCs by Aq.F was in a dose-dependent manner; IC_{50} followed by IC_{25} have significantly inhibited cell migration more than IC_{10} ($p < 0.05$). On the contrary, potent fractions (Bu.f and CEE) demonstrated a different mode of response; migration inhibition by IC_{25} was higher than IC_{10} in both fractions ($p < 0.05$). However, the highest concentration (IC_{50}) of Bu.f and CEE produced the lowest cell migration inhibition amongst all other concentrations (Fig. 5).

Butanol fraction and CEE induced a different pattern of the cellular migratory reaction in the chemotaxis assay compared to the aqueous fraction. Unexpectedly, the addition of high concentration (IC_{50}) of Bu.f or CEE to cell culture media increased the migration more than other concentrations. This can be explained as the high dose increases oxidative stress due to increasing H_2O_2 generated *in vitro* in a dose-dependent fashion. High antioxidants' concentration and the multiplicity of their compounds supposedly augment H_2O_2 concentration in cell culture so that they exceeded their antioxidant role and react as pro-oxidants.[32] Furthermore, some flavonoids such as catechin, quercetin, and epigallocatechin were found to generate more H_2O_2 than others when added to cell culture media,[33] and those flavonoids were previously screened in *C. caudatus* extracts.[8,13] Moreover, it has been found that many polyphenols are not stable in DMEM, which is the culture media used in this study, and this increases the chance for hydrogen peroxide formation *in vitro*.[34] Aqueous fraction is considered the fraction with the lowest phenolics and saponins' concentration among *C. caudatus* fractions in this study. When cells treated with Aq.f at IC_{50}, they showed the highest level of cell migration inhibition in the chemotaxis assay. This can be interpreted by that the concentration of phenolics and saponins found in this fraction was exactly enough to inhibit cell migration more than other fractions at the same concentration. Thus, the concentration of H_2O_2 might not reach the level whereby the antioxidants converted to pro-oxidants.

In order to prove that cell migration reduction was not caused by reduced cell viability, cells in inserts containing *C. caudatus* extract/fractions were tested with trypan blue exclusion assay to check their viability after the migration assay. In an unpublished data, 70–80% of cells were still viable after 6 h of incubation with fractions/extract. Moreover, in crude and butanol fractions-containing inserts, more cells migrated to the lower chamber when they were treated with the higher concentration (IC_{50}) compared with cells treated with the intermediate one (IC_{25}). This means that cell viabilities have not been affected by treatments' cytotoxicity due to that cells were exposed to treatments only for 6 h, and more importantly, it seems that cells had migrated away from the highly-concentrated treatment in the first few hours before getting affected by it.

Chemoinvasion inhibition activity

A-10 cell invasion inhibition activity for *C. caudatus* extract/fractions at three different concentrations is determined by this assay. Unlike migration assay, all fractions followed the same trend with increasing treatment concentrations. The IC_{25} dose showed significantly higher cell invasion inhibition than IC_{10} ($p < 0.05$). However, when cells exposed to high doses at IC_{50}, their invasion inhibition activity decreased. Similar to cell behavior in the chemotaxis assay, cell invasion inhibition maximum capacity was at the intermediate concentration (IC_{25}) for the three extract/fractions (Fig. 6).

Due to that cell incubation with treatments in chemoinvasion assay was for 24 h, we had to prove that cell invasion reduction was not because of cell viability reduction. Chemoinvasion assay detects cells that already performed the invasion process by counting them on the lower surface of the polycarbonate membrane facing the lower chamber. It is obvious that more cells invaded toward the lower chamber in the highest concentration-containing inserts (IC_{50}) compared to mild (IC_{10}) and intermediate (IC_{25}) concentrations-containing inserts. This proposes that cells exposed to IC_{50} had instantly invaded away from it in the first few hours of incubation and before its viability reduced by the treatment. The in vivo/in vitro discrepancies are well-established especially in terms of cell membrane permeation of drugs and phytochemicals.[35] However, cells in culture are less suitable to express oxidative stress suppression by antioxidants due to over-production of some molecules and signaling factors such as reduced

NADP and pyruvate *in vitro*.[36] Therefore, such work can reflect a partial image of the effects of phytochemicals on cell migration and invasion and further *in vivo* study is needed to confirm these results.

The difference between migration and invasion assays in cell behavior when treated with the aqueous fraction can be attributed to cell properties that make it able to respond to a chemotactic stimuli by migration or invasion. Cells ability to form contractile filaments and microtubules in order to migrate is different from properties needed for them to invade a basement membrane layer such as the activation of metalloproteinases enzymes.[37] Besides, diverse treatments and oncogene transfections can possibly affect only one of these properties.[38] For instance, Garbisa et al.[39] found that epigallocatechin-3-gallate has reduced tumor cell invasion activity but not the migration one.

Cells in culture respond to H_2O_2 differently according to the cell line and H_2O_2-neutralizing substances produced by these cells such as the catalase enzyme.[33] H_2O_2 enhances oxidative stress through the activation of transcription factors such as NF-κB and AP-1 and consequently increases expression of adhesion molecules VCAM, ICAM, and E-selectin.[40] In this study, high polyphenolics concentration can be considered the key factor that potentiates cell adhesion on the ECM layer and subsequently a higher invasion rate. Moreover, it has been found that H_2O_2 directly increases the expression of c-Jun as part of its mechanism as an oxidant.[41] The up-regulation of the c-Jun NH2-terminal kinase (JNK) signaling pathway activates the transcription factor c-Jun, and the latter increases MMP-2 production and consequently increases cell invasion and migration.[42]

Invasion inhibition index

In vivo, cell migration and invasion are inseparable, therefore, cell ratio that has the ability to invade the ECM layer to the general cell mobility should be determined. In order to do so, we employed the concept of "the invasive index".[37] This study targets atherosclerosis reduction power of extract/fractions derived from *C. caudatus*, where we measure their inhibition to smooth muscle cells migration and invasion *in vitro*. Therefore, we employed the following formula to calculate the invasion inhibition index.

$$Invasion\ Inhibition\ Index = 100 - \left(\left(\frac{OD\ invaded\ cells}{OD\ migrated\ cells} \right) \times 100\% \right)$$

Fig. 7 shows close index values for butanol and crude fractions, while aqueous fraction followed a different trend. This reflects the similarity between crude and butanol fractions constituents and activities. IC_{25} was the concentration that all three fractions were in close index values. Unexpectedly, Bu.f and CEE at the lowest concentration at (IC_{10}) showed the highest ratio of cell invasion to the total cell migration inhibition, 68.05%, and 68.28% respectively. This indicates that when a closer-to-*in vivo* system was applied, a lower treatment concentration had achieved a higher cell invasion to migration inhibition ratio and possibly, a more beneficial effect in atherosclerosis reduction.

Conclusion

The present study was designed to determine the antioxidant and cell migration and invasion inhibitory *in vitro* activities of *C. caudatus* extracts and derived fractions on vascular smooth muscle cells. In addition to that, it provides a comparison between effects of these extract/fractions as well as the correlation between their phytochemical contents and antioxidant activities. Despite the fact that this study did not screen phytochemicals that are available in each fraction, it offered an insight into potential effects of *C. caudatus* as an atheroprotective herb that is also used in many aspects of folk medicine in tropical areas.

Firstly, this study has revealed a cost effective extraction method by using aqueous-butanol fractionation system, which piles up high contents of saponins and phenolics in the butanol fraction. Secondly, since Bu.f comprises more phenolics, saponins, and steroidal saponins contents, it showed the strongest activities in all antioxidant assays, besides, it did decrease cell viability in MTT assay more efficiently than other fractions. Thirdly, high concentrations of all fractions demonstrated pro-oxidant effects characterized by increasing migration and invasion rate of the VSMCs, while the lowest concentration of the butanol and crude fractions were better in invasion inhibition index. At the same time, intermediate concentration (IC_{25}) for the three fractions showed the highest cell invasion inhibition. Finally, *Cosmos caudatus* presents a promising potential for developing new leads that can be used in atherosclerosis suppression.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbj.2017.03.008.

References

1. Marín-García J. Molecular determinants of atherosclerosis. In: Marín-García J, editor. Post-genomic cardiology. Second Edition Boston: Academic Press; 2014. p. 183–215.
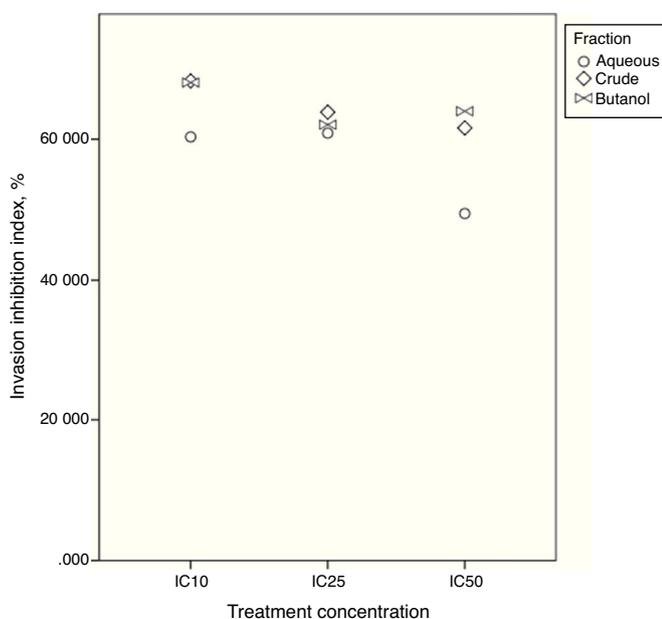2. Catapano AL, Wiklund O. Think again about cholesterol survey. Atheroscl Suppl. 2015;20:1–5.

Fig. 7. Invasion inhibition index for the aqueous, crude and butanol extract/fractions of *C. caudatus* at different doses.

3. Louis SF, Zahradka P. Vascular smooth muscle cell motility: from migration to invasion. Exp Clin Cardiol. 2010;15:e75–85.
4. Ionita MG, Arslan F, De Kleijn DP, Pasterkamp G. Endogenous inflammatory molecules engage Toll-like receptors in cardiovascular disease. J Innate Immun. 2010;2:307–15.
5. Quattrocchi U. CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology. 5th ed. New York: CRC Press; 2012.
6. Gibbs H, Agcaoili F. The alcohol industry of the Philippine islands. Part 3. Fermented beverages which are not distilled. Philipp J Sci. 1912;7:97–121.
7. Hassan WW, Mahmood M. Healing herbs of Malaysia. 2nd ed. Kuala Lumpur: Biotropics Malaysia Berhad; 2007.
8. Abas F, Lajis N, Kalsom YU. Antioxidative and radical scavenging properties of the constituents isolated from Cosmos caudatus Kunth. Nat Prod Sci. 2003;9:245–8.
9. Andarwulan N, Kurniasih D, Apriady RA, Rahmat H, Roto AV, Bolling BW. Polyphenols: carotenoids, and ascorbic acid in underutilized medicinal vegetables. J Funct Foods. 2012;4:339–47.
10. Javadi N, Abas F, Hamid AA, Simoh S, Shaari K, Ismail IS, et al. GC-MS-based metabolite profiling of Cosmos caudatus leaves possessing alpha-glucosidase inhibitory activity. J Food Sci. 2014;79. C1130-C6.
11. Mediani A, Abas F, Khatib A, Tan CP. Cosmos caudatus as a potential source of polyphenolic compounds: optimisation of oven drying conditions and characterisation of its functional properties. Molecules. 2013;18:10452–64.
12. Mustafa R, Hamid AA, Mohamed S, Bakar FA. Total phenolic compounds: flavonoids, and radical scavenging activity of 21 selected tropical plants. J Food Sci. 2010;75:C28–35.
13. Shui G, Leong LP, Wong SP. Rapid screening and characterisation of antioxidants of Cosmos caudatus using liquid chromatography coupled with mass spectrometry. J Chromatogr B. 2005;827:127–38.
14. Loh SP, Hadira O. In vitro inhibitory potential of selected Malaysian plants against key enzymes involved in hyperglycemia and hypertension. Malays J Nutr. 2011;17:77–86.
15. Perumal V, Hamid AA, Ismail A, Saari K, Abas F, Ismail IS, et al. Effect of Cosmos caudatus kunth leaves on the lipid profile of a hyperlipidemia-induced animal model. J Food Chem Nutr. 2014;2:43–51.
16. Chan KW, Khong NM, Iqbal S, Ismail M. Isolation and antioxidative properties of phenolics-saponins rich fraction from defatted rice bran. J Cereal Sci. 2013;57:480–5.
17. Chan KW, Iqbal S, Khong NM, Ooi D-J, Ismail M. Antioxidant activity of phenolics–saponins rich fraction prepared from defatted kenaf seed meal. LWT-Food Sci Technol. 2014;56:181–6.
18. Hiai S, Oura H, Nakajima T. Color reaction of some sapogenins and saponins with vanillin and sulfuric acid. Planta Med. 1976:116–22.
19. Baccou J, Lambert F, Sauvaire Y. Spectrophotometric method for the determination of total steroidal sapogenin. Analyst. 1977;102:458–65.
20. Chan KW, Khong NM, Iqbal S, Mansor SM, Ismail M. Defatted kenaf seed meal (DKSM): prospective edible flour from agricultural waste with high antioxidant activity. LWT-Food Sci Technol. 2013;53:308–13.
21. Wettasinghe M, Shahidi F. Antioxidant and free radical-scavenging properties of ethanolic extracts of defatted borage (Borago officinalis L.) seeds. Food Chem. 1999;67:399–414.
22. Abas F, Lajis NH, Israf D, Khozirah S, Kalsom YU. Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables. Food Chem. 2006;95:566–73.
23. Chukwumah YC, Walker LT, Verghese M, Ogutu S. Effect of frequency and duration of ultrasonication on the extraction efficiency of selected isoflavones and trans-resveratrol from peanuts (Arachis hypogaea). Ultrason Sonochem. 2009;16:293–9.
24. Oleszek W, Hamed A. Saponin-based surfactants. Surf Renewable Res. 2010:239.
25. Kerem Z, German-Shashoua H, Yarden O. Microwave-assisted extraction of bioactive saponins from chickpea (Cicer arietinum L). J Sci Food Agric. 2005;85:406–12.
26. Sulaiman SF, Ooi KL. Antioxidant and anti food-borne bacterial activities of extracts from leaf and different fruit parts of Myristica fragrans Houtt. Food Control. 2012;25:533–6.
27. Francisco-Marquez M, Aguilar-Fernández M, Galano A. Anthranilic acid as a secondary antioxidant: implications to the inhibition of OH production and the associated oxidative stress. Comp Theor Chem. 2016;1077:18–24.
28. Chia YY, Kanthimathi M, Khoo KS, Rajarajeswaran J, Cheng HM, Yap WS, Antioxidant and cytotoxic activities of three species of tropical seaweeds. BMC Complement Altern Med. 2015;15:339.
29. Neubig RR, Spedding M, Kenakin T, Christopoulos A. International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on terms and symbols in quantitative pharmacology. Pharmacol Rev. 2003;55:597–606.
30. Boyd MR. The NCI in vitro anticancer drug discovery screen. In: Teicher BA, editor. Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials and Approval. New York: Springer+Business Media; 1997. p. 23–42.
31. Cruz MS, Cabral Barroso S, Navoni JA, Rocha Silva Teles MM, Barbosa-Filho JM, de Oliveira Rocha HA, et al. Effect of hecogenin on DNA instability. Tox Rep. 2016;3:539–43.
32. Lapidot T, Walker MD, Kanner J. Can apple antioxidants inhibit tumor cell proliferation? Generation of H_2O_2 during interaction of phenolic compounds with cell culture media. J Agric Food Chem. 2002;50:3156–60.
33. Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun. 2000;273:50–3.
34. Long LH, Hoi A, Halliwell B. Instability of: and generation of hydrogen peroxide by, phenolic compounds in cell culture media. Arch Biochem Biophys. 2010;501:162–9.
35. Kato Y. Overcoming in vitro/in vivo discrepancies. Drug Metab Pharmacokinet. 2009;24:409–10.
36. Halliwell B. Free radicals and antioxidants: updating a personal view. Nutr Rev. 2012;70:257–65.
37. Albini A, Benelli R. The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation. Nat Protoc. 2007;2:504–11.
38. Albini A, Iwamoto Y, Kleinman H, Martin G, Aaronson S, Kozlowski J, et al. A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. 1987;47:3239–45.
39. Garbisa S, Biggin S, Cavallarin N, Sartor L, Benelli R, Albini A. Tumor invasion: molecular shears blunted by green tea. Nat Med. 1999;5:1216–22.
40. Vogiatzi G, Tousoulis D, Stefanadis C. The role of oxidative stress in atherosclerosis. Hellenic J Cardiol. 2009;50:402–9.
41. Zhang J, Jin N, Liu Y, Rhoades RA. Hydrogen peroxide stimulates extracellular signal-regulated protein kinases in pulmonary arterial smooth muscle cells. Am J Respir Cell Mol Biol. 1998;19:324–32.
42. Cheung LW, Leung PC, Wong AS. Gonadotropin-releasing hormone promotes ovarian cancer cell invasiveness through c-Jun NH2-terminal kinase-mediated activation of matrix metalloproteinase (MMP)-2 and MMP-9. Cancer Res. 2006;66:10902–10.